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Video Article

Cytotoxic Efficacy of Photodynamic Therapy in Osteosarcoma Cells *In Vitro*

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Abstract

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Photodynamic Therapy (PDT) that makes use of a nontoxic photosensitizer (PS), which, upon activation with light of a specific wavelength in the presence of oxygen, generates oxygen radicals that elicit a cytotoxic response[1]. Despite its approval almost twenty years ago by the FDA, PDT is nowadays only used to treat a limited number of cancer types (skin, bladder) and nononcological diseases (psoriasis, actinic keratosis)[2].

The major advantage of the use of PDT is the ability to perform a local treatment, which prevents systemic side effects. Moreover, it allows the treatment of tumors at delicate sites (*e.g.* around nerves or blood vessels). Here, an intraoperative application of PDT is considered in osteosarcoma (OS), a tumor of the bone, to target primary tumor satellites left behind in tumor surrounding tissue after surgical tumor resection. The treatment aims at decreasing the number of recurrences and at reducing the risk for (postoperative) metastasis.

In the present study, we present *in vitro* PDT procedures to establish the optimal PDT settings for effective treatment of widely used OS cell lines that are used to reproduce the human disease in well established intratibial OS mouse models. The uptake of the PS mTHPC was examined with a spectrophotometer and phototoxicity was provoked with laser light excitation of mTHPC at 652 nm to induce cell death assessed with a WST-1 assay and by the counting of surviving cells. The established techniques enable us to define the optimal PDT settings for future studies in animal models. They are an easy and quick tool for the evaluation of the efficacy of PDT *in vitro* before an application *in vivo*.

Video Link

The video component of this article can be found at http://www.jove.com/video/51213/

Introduction

Today's state of the art treatment of osteosarcoma (OS), a primary bone tumor, encompasses a combination of neo adjuvant chemotherapy and surgery. This treatment regimen revealed an increase in the survival rate of patients with localized disease from approximately 20% before the use of chemotherapy, to currently between 60-70%[3,4]. However, in the last two decades, the overall survival of OS patients with local disease has plateaued[4,5]. Moreover, 30-40% of these patients relapse within 3 years after diagnosis and patients with metastatic disease continue to have a poor survival of 20-30%[4,6,7]. To improve the outcome of these patients, new therapeutic strategies need to be developed.

Photodynamic Therapy (PDT), a rather novel anticancer therapy, uses light of a specific wavelength for excitation of a photosensitizer (PS), which accumulates in the tumor cells after its injection into the bloodstream. Laser light excitation of the PS generates oxygen radicals in the presence of oxygen, which induce cytotoxic reaction in tumor cells and cell death. Besides this primary mechanism, two additional PDT evoked biological processes contribute to reduced tumor growth: PDT causes vasoconstriction and thrombus formation of the tumor microvasculature and, consequently local hypoxia and anoxia inside the tumor, leading to tumor infarction. Finally, PDT injured and dying tumor cells trigger a local immune response, a rather unique feature of PDT. This involves the complement system and the activation of antigen presenting dendritic cells[8]. Thus, conditions are created for the presentation of tumor antigens with subsequent activation of lymphoid cells, leading to tumor specific immunity.

So far, PDT has been used to treat several types of soft tissue tumors and hyperplasia's, such as actinic keratosis, Barrett's esophagus, endobronchial tumors, bladder cancer, basal cell carcinomas, and palliative treatment of head and neck cancer[2]. The treatment is known to induce local, large scale necrosis with only little side effects, and thus has the potential to selectively eradicate tumor tissue. Despite these advantages, the application of PDT remains technically more demanding than the administration of chemotherapeutic drugs. In order to achieve maximal efficacy, the PS concentration, light exposure time and total light energy transfer need to be optimized. This can be done in *in vivo*

experiments, but, because of the relative large number of parameters that need to be optimized, it is more efficient to initially determine optimal conditions *in vitro*.

In the experiments described below, we tested the *in vitro* efficacy of PDT using the PS 5,10,15,20-tetrakis(meta-hydroxyphenyl)chlorin, abbreviated mTHPC (**Figure 1A**). mTHPC is the active substance in the medicinal product Foscan, which is currently used in the clinic for palliative treatment of head and neck cancer. It is one of the most potent PS, inducing massive cell damage already at low concentrations, and it was demonstrated to be superior to other PS in terms of tissue penetration[9,10]. Its light absorption spectrum (**Figure 1B**) shows two prominent peaks, one at 417 nm and a second at 652 nm, which are used for tissue localization of accumulating PS and for PDT induction, respectively.

Currently, a liposomal formulation for mTHPC is under development. Here, we describe the procedures to quantify the uptake of this liposomal formulation, and to perform PDT in two human OS cell lines; the low metastatic HOS and the high metastatic 143B cells. Some of the data presented here have been reported earlier[11]. The approach described here enables us to study the effect of a metastatic phenotype on PDT efficacy. 143B cells, orthotopically injected into the hind limbs of immune deficient SCID mice cause intratibial metastasizing primary tumors, a model closely mimicking the human metastasizing disease. Thus, the proposed *in vitro* experiments are perfectly suitable to assess the optimal PDT settings to be later used in *in vivo* experiments.

Protocol

1. Comparison of the Uptake of mTHPC in the Respective Low and Highly Metastatic HOS and 143B OS Cell Lines

1. Prepare cell culture medium containing DMEM, Ham F12, and heat inactivated fetal calf serum in a ratio 4.5:4.5:1.

   Note: The liposomal formulation of the PS mTHPC was dissolved in water at a final concentration of 1.5 mg/ml mTHPC.
2. Plate 0.2 x 10^6 HOS and 143B cells/well in 6-well plates (triplicates for each condition) and let the cells adhere overnight.

   Note: the number has to be adjusted to individual cell lines. A confluency of 80-90% is most suitable at the time of the experiment.
3. On the following day, incubate the cells with a fixed concentration of mTHPC for different time periods (time dependent uptake), or with increasing concentrations of mTHPC during a fixed time period (dose dependent uptake).
4. Incubate the cells with mTHPC for specific time points or concentrations of mTHPC.

   Note: In this study, both the HOS and 143B cell lines were incubated with 0.6 µg/ml mTHPC for 0, 2.5, 5, or 10 hr, or with 0, 0.6, 2.5, and 10 µg/ml mTHPC for 5 hr. The applied time periods and dosages may vary depending on the cell line that is used.
5. Remember to always work in the dark or in dimmed light conditions to prevent direct light exposure of the cells.
6. After incubation of the cells at the indicated conditions, aspirate the medium and wash the cells three times with PBS. Next, detach the cells with 0.5 ml trypsin/EDTA and count the cells.
7. After detachment, centrifuge the cells at 400 x g for 5 min, aspirate the medium and resuspend the cells in PBS to a final density of 200,000 cells/ml.
8. Pipette 100 µl of the obtained cell suspension (*i.e.* 20,000 cells) in a 96-well plate and measure the fluorescence of these cells in a fluorescence spectrophotometer . The settings for mTHPC fluorescence measurements are: 417 nm for the excitation and 652 nm for the emission.
9. In order to calculate the amount of internalized mTHPC per cell, normalize the relative fluorescence unit (RFU) for the cell number and cell volume as follows:
10. Prepare a standard curve using different concentrations of mTHPC in PBS from 0-4 µg/ml (use triplicate measurements). Pipette 100 µl of each dilution in a 96-well plate and measure the fluorescence of the wells using the settings described under step 1.6.
11. Divide the RFU (obtained for 20,000 cells) by the slope of the linear standard curve of mTHPC, which gives the concentration of mTHPC (in µg/ml) in the well.
12. Divide this mTHPC concentration by 10 to get the total amount of mTHPC in one well (in µg, representing 100 µl of cell suspension) and divide this value by the volume of 20,000 cells.
13. Calculate the volume with the formula for a sphere: (4/3 x π) x r^3. Obtain the radius *r* by measuring the diameter of 20 trypsinized cells under a microscope, and dividing this value by 2.
14. Finally, normalize all the values to those obtained for HOS cells after 10 hr of incubation (dose dependent uptake) or treated with 10 µg/ml mTHPC (time dependent uptake), which were set to 100%.

2. Measurement of Phototoxicity of PS *In Vitro*

1. Seed 3,000 cells/well in a 96-well plate (triplicates for each concentration of mTHPC used) and let them adhere overnight. Similarly prepare an additional 96-well plate and treat the cells as described below, but then put them aside for phase contrast imaging.

   Note: the number has to be adjusted for each individual cell line. A confluency of 50-60% at the time of the experiment is ideal.
   1. Keep in mind to add the corresponding dark toxicity control of cells for each of the mTHPC concentrations. These are cells that will be incubated in the absence or presence of selected concentrations of mTHPC, but will not be illuminated.
2. On the following day, incubate the cells with different concentrations of mTHPC (0, 0.0001, 0.001, 0.01, 0.03, 0.075, 0.15, 0.3, 0.6, 1.25, 2.5, 5, and 10 µg/ml) depending on the cell lines and keep them in the dark for 5 hr.
   1. Refer to Section 1.3.2

Note: The incubation time before illumination depends on the cell line and on the PS used, therefore it is advisable to test different concentrations and different time points in separate experiments.

3. Following incubation for the indicated time period, wash the cells twice with PBS and add new fresh medium without mTHPC.
4. Illuminate the cells with a 652 nm diode laser, specific for the mTHPC absorption spectrum (see **Figure 1**).
    1. Set the laser to 21.88 mW/cm^2 power at a height of 13.5 cm distance from the cells, in order to get an energy dose of 5 J/cm^2. Illuminate the cells for 230 sec. Do not illuminate the dark toxicity control cells (with and without mTHPC).

    Note: Always wear glasses to protect the eyes from the laser light used for excitation of the PS.

5. After illumination, keep the cells in the dark at 37 ºC for 24 hr. Subsequently, add water soluble tetrazolium (WST-1) reagent (10 µl/100 µl of medium).
6. Three hours after the addition of the WST-1 reagent, measure the absorbance of individual wells of the 96-well plate at 415 nm.
7. In order to calculate the percentage of surviving cells, set the mean of the values obtained for the non mTHPC treated cells for each condition to 100% (*i.e.* illuminated, without mTHPC and nonilluminated, without mTHPC).

3. Estimation of Cell Number by Cell Counting

1. Seed 20,000 cells/well in a 24-well plate and let them adhere overnight.
2. Incubate the cells for 5 hr with mTHPC (0.001, 0.003, 0.15, 0.6, and 1.25 µg/ml) and illuminate them as described above.
3. Collect the medium, wash the cells once with PBS, trypsinize them and collect them by centrifugation at 400 x g for 5 min.
4. After centrifugation, aspirate the medium and resuspend the cells in 200 µl of fresh medium.
5. Count the cells in a Neubauer chamber.

Representative Results

With the here reported techniques, we investigated mTHPC-based PDT in human OS cells. First, the time and dose dependent uptake of mTHPC was investigated in the low metastatic HOS and in the highly metastatic 143B OS cell lines. mTHPC uptake can be assessed by measuring the fluorescence of mTHPC with a fluorescence spectrophotometer (**Figure 2**, reproduced with permission from Reidy *et al.*[11]). **Figure 2A** illustrates the uptake of mTHPC in a time dependent manner. The fluorescence intensity of the cell suspension represents the intracellular levels of mTHPC. **Figure 2B** illustrates the dose dependent mTHPC uptake in HOS and 143B cells after 5 hr incubation. The uptake in the high metastatic cell line 143B tended to be higher than in the low metastatic parental HOS cell line.

Dark and phototoxicity of mTHPC in 143B cells was assessed by determining the cell metabolic activity (with a WST-1 assay) and by counting the number of residual cells after 5 hr of incubation with mTHPC, subsequent incubation in the dark or treatment with laser light for 230 sec and further incubation for 24 hr in the dark.

Dose dependent dark toxicity is shown in **Figure 3** (reproduced with permission from Reidy *et al.*[11]). In **Figure 3A**, 143B cells were treated with different mTHPC dosages and compared to untreated cells. A dose-dependent decrease of cell number and metabolic activity was observed. Decreased cell viability was recognized at an mTHPC concentration equal and higher than 2.5 µg/ml. Dose dependent phototoxicity of mTHPC is illustrated in **Figure 3B**. Cells were treated with different mTHPC doses and with subsequent illumination (5 J/cm^2). A consequential decrease in cell metabolic activity (measured with the WST assay), as well as a decrease in cell number was observed. The resulting decrease in cell number after applying PDT is visualized in **Figure 4**.

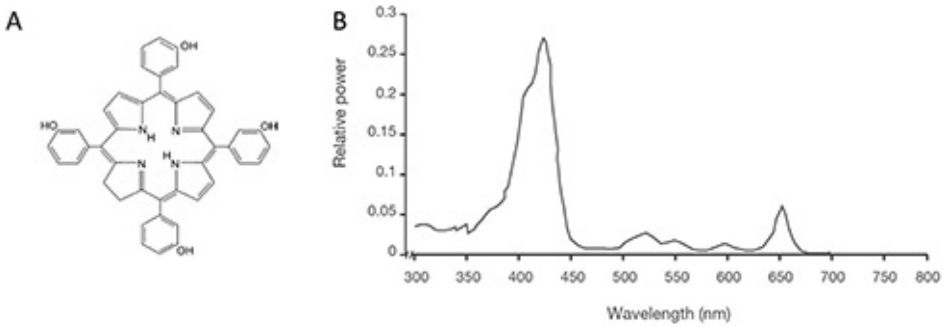

Figure 1. Chemical structure of mTHPC (A) and light absorption spectrum (B). Figures were provided by biolitec Research GmbH. Please click here to view a larger version of this figure.

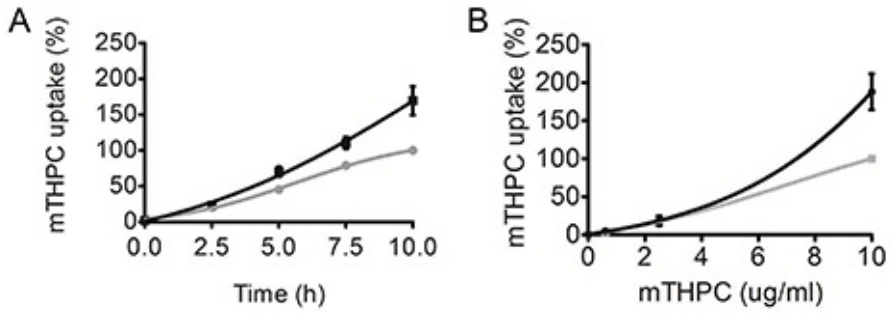

Figure 2. Time and dose dependent uptake of mTHPC by the human OS cell lines HOS and 143B. (**A**). HOS (in grey) and 143B (in black) were seeded in 6-well plates (0.2 x 20^6 cells/well). After overnight culturing, the cells were incubated with 0.6 µg/ml mTHPC for 0, 2.5, 5, 7.5, and 10 hr (**A**), or for 5 hr with 0, 0.6, 2.5, and 10 µg/ml mTHPC **(B)**. Fluorescence intensity was determined by measuring the fluorescence of 20,000 cells at 652 nm upon excitation at 417 nm. Values were normalized to the calculated cell volume and the mTHPC uptake of HOS cells at 10 hr (**A**) or after incubation with 10 µg/ml (**B**) was set to 100%. Values are the mean ± SEM of three independent experiments. Adapted from Reidy *et al.*[11] Please click here to view a larger version of this figure.

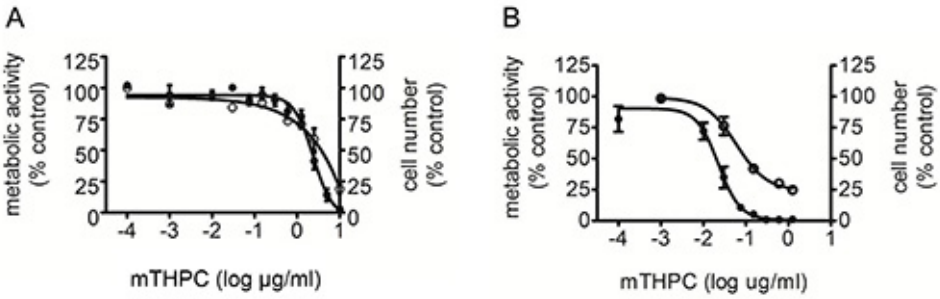

Figure 3. Dark and phototoxicity of mTHPC in 143B cells. Cells were seeded and grown overnight before treatment. To determine mTHPC dose dependent dark toxicity, 143B cells were incubated for 5 hr in the dark in the absence or presence of indicated mTHPC concentrations (**A**). To determine mTHPC dose dependent photo toxicity, 143B cells were incubated with or without mTHPC for 5 hr at indicated concentrations and then illuminated with 5 J/cm^2 with a power of 21.88 mW/cm^2 for 230 sec (**B**). For measurements of the metabolic activity (●) with the WST-1 assay, 143B cells were seeded in 96-well plates at a density of 3,000 cells/well. Cell numbers (○) were counted from cells seeded at 20,000 cells/well in 24-well plates. Values are the mean ± SEM of three independent experiments. Adapted from Reidy *et al.*[11] Please click here to view a larger version of this figure.

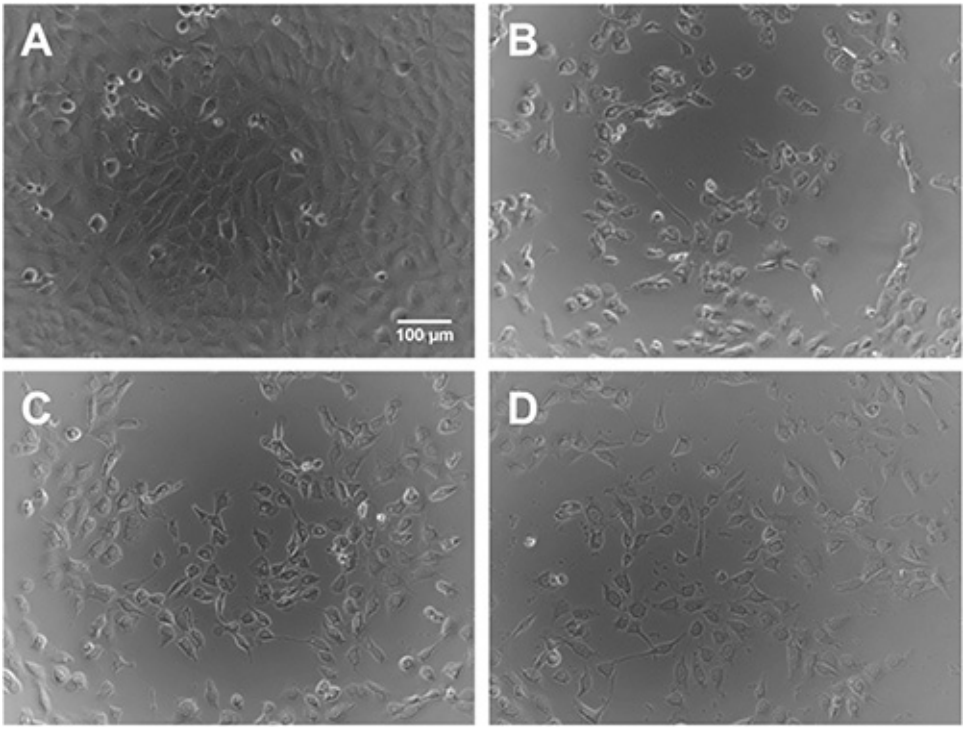

Figure 4. Phase contrast images of 143B cells before and after PDT. Cells were seeded and left to adhere overnight as described in the protocol. To visualize dose dependent, mTHPC mediated light toxicity, 143B cells were incubated for 5 hr in the dark with 0, 0.6, 2.5, and 10 µg/ml mTHPC (panels A-D, respectively). The cells were then illuminated with 5 J/cm^2 light of 652 nm wavelength for 230 sec, and photographed after 24 hr. Scale bar: 100 µm. Please click here to view a larger version of this figure.

Discussion

To achieve optimal cytotoxicity in response to PDT, it is crucial to choose the right laser light settings and incubation times. The here described procedures are consistent and efficient to determine PS uptake and to quantify PDT induced cytotoxicity *in vitro*. Using the specific absorption wavelengths of the PS mTHPC, the cellular PS uptake can be determined in a direct manner, and the PS can be activated to generate cytotoxic reactive oxygen species.

Using this *in vitro* setup, all parameters such as PS concentration, drug light interval and laser light intensity can be easily adjusted to the characteristics of the PS and the cell lines used. Cell count gives a direct read out of surviving attached cells, which together with the WST-1 assay (monitoring metabolic activity) gives a good estimate for cell survival. However, the presented method can only model the “first hit”, *i.e.* the cell toxicity of reactive oxygen species immediately after activation of the PS. The *in vivo* observed subsequent vasoconstriction and the activation of the immune system, necessary for full tumor eradication, can only be investigated in animals.

Figure 2 demonstrated a time and dose dependent uptake of mTHPC to a higher level in the highly metastatic 143B than in its parental low metastatic HOS cell line. Dark toxicity of mTHPC in 143B OS cells was observed after incubation for 5 hr only at high concentrations (**Figure 3**). 143B cells show a high sensitivity to PDT, shown by the mTHPC and light dose dependent phototoxic effect. A phototoxic effect was observed already at an mTHPC concentration as low as 0.075 µg/ml, and a half maximal lethal concentration of 0.022±0.006 µg/ml was observed (**Figure 3**).

Although PDT is minimally invasive and efficient against many tumor types, minor side effects can occur when applying the technique in patients (*e.g.* swelling, erythema). This indicates that the PS is not only taken up by tumor cells, as shown here, but also by nontumor cells, causing toxicity after PDT also in these cells. Triesscheijn *et al.* was able to show an increased photosensitivity of tumor cells compared to human fibroblasts. In contrast, human microvascular endothelial cells showed a higher uptake of mTHPC than the other tested cell lines, which led to a high photosensitivity[12]. These findings also correlate with PDT associated anti vascular effects, which are frequently observed[2].

The described methods can also be applied for other PS. Incubation time and laser light intensity need to be adapted to the characteristics of the PS. Additionally, PDT can also be induced in other than OS cells, since only the settings are cell line dependent but not the method itself. As additional read outs, cell death mechanisms can be analyzed, *e.g.* by Western Blot analysis of proteins involved in apoptosis, necrosis or autophagy. Also the subcellular localization of PS can be visualized *e.g.* by confocal laser scanning microscopy.

The different steps that have been described for PDT *in vitro*, *i.e.* incubation of the cells with the PS and induction of PDT with laser light, closely resemble the clinical situation. Patients receive (i.v. or s.c. applied) the PS, and after a distinct drug light interval, the area of interest (*e.g.* tumor tissue) is illuminated with laser light[13]. Although not yet applied in OS, its potential advantages are obvious; PDT is considered to be particularly attractive for the intraoperative treatment of tumor satellites, which frequently grow as primary tumor cell clusters close to the main rim of the primary tumor and may initiate a recurrence when missed during surgical resection of the primary tumor. Even large osseous tumors may be

treated with this technique. This has been shown by a promising study in dogs[14]. In addition, eradication of tumor cells that cannot be resected by surgery (*e.g.* multiple metastases mostly occurring in the lungs), may also represent a possible future application of PDT in patients with OS metastatic disease.

It is important to note that, while performing the experiments, care should be taken that all working steps are performed under minimal light exposure after adding the PS to the cells, as normal daylight also contains the wavelength necessary to activate mTHPC. Likewise, for *in vivo* application in preclinical models, care needs to be taken to shield the animals from direct light after injecting mTHPC. It also needs to be noted that the WST-1 assay only assesses the metabolic activity of tumor cells, which is an indirect indicator of cell death. Therefore, a combination with a total cell count, as presented here, is recommended.

In conclusion, the combination of the described methods (PS-uptake and PDT-induced cell toxicity) provides a fast and cost effective procedure to characterize toxicity properties of a PS *in vitro*. With these *in vitro* results, the range of the optimal light intensity and the drug light interval can be estimated, which gives a good indication of how PDT can be applied *in vivo*.

Disclosures

The authors declare no competing financial interests or conflicts of interest.

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